Findings From ALTS

Impact on Cervical Cytology Screening, Triage, and Patient Management

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Objective: To summarize some of the main results of the ASCUS/LSIL Triage Study (ALTS), a randomized multicenter clinical trial to compare management strategies for women with ASCUS and LSIL cytologic findings.

Study Design: A total of 5060 women, 3488 with an ASCUS (atypical squamous cells of undetermined significance) and 1572 with LSIL (low-grade squamous intraepithelial lesion) cytology, were randomly assigned to 1 of 3 arms: (1) immediate colposcopy regardless of enrollment test results; (2) testing for human papillomavirus (HPV) DNA with referral to colposcopy for a positive result; and (3) conservative management based on repeat cytology with HSIL (high-grade squamous intraepithelial lesion) as the threshold for referral to colposcopy. All arms included 2 years of semiannual follow-up and colposcopy at exit for patient safety and disease ascertainment.

Results: For women with ASCUS, the cumulative detection of CIN3 and cancer (CIN3+) was 8–9% over 2 years of follow-up and did not vary significantly by study arm. A single HPV test at enrollment was positive in 92% of the cases of CIN3+ and was positive in 53% of women overall. Two repeat cytology evaluations, with a low referral threshold of ASCUS+, demonstrated high sensitivity of 95% for CIN3+ but would result in 67% of women sent to colposcopy. The strategy of immediate colposcopy for all women detected only about half of the cumulative (2-year) cases of CIN3+.

For women with LSIL, the cumulative detection of CIN3+ was 15.0% and did not vary significantly by arm. Of note, the detection of CIN3+ for LSIL was similar to the risk for HPV-positive ASCUS, 15.2%, supporting a unified view of the 2 interpretations. No useful triage test strategy was identified for LSIL, which was predominantly HPV positive (83%) by Hybrid Capture 2 (HC2). Some LSIL was associated with nononcogenic HPV types by PCR testing. Only 5–10% of LSIL was HPV negative by both HC2 and PCR; these cases included instances of false-negative HPV test results or false-positive cytologic interpretations of LSIL.

ALTS analyses have confirmed the 1.0 RLU/PC positive cutpoint for HC2 as the most appropriate for clinical utility. HC2 was more reproducible with an overall κ of 0.84 compared to liquid based cytology interpretation and histologic diagnosis of colposcopically directed biopsies, both of which demonstrated a κ of 0.46.

Conclusion: The ALTS findings have informed the development of evidence-based management guidelines for women with ASCUS and LSIL cytology. HPV triage is a cost-effective management approach for women with ASCUS and spares approximately half of women the need for a colposcopic examination. Repeat cytology requires multiple follow-up vists to achieve similar sensitivity as HPV testing. There was no effective triage identified for women with LSIL cytology; consequently guidelines recommend evaluation by colposcopy. The prospective follow-up of ALTS participants provided the opportunity to identify missed prevalent disease and demonstrated that no one test was completely sensitive for detection of CIN3+. All tests, including colposcopically directed biopsy (previously considered the "gold standard"), have limitations.

Key Words: cervix, cervical cytology, HPV, triage, colposcopy

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In 1988, the Bethesda System introduced the term *atypical squamous cells of undetermined significance* (ASCUS) to communicate equivocal morphologic findings in cervical cytology.[1] Prior to the use of ASCUS, ambiguity in cytologic interpretation existed but was often couched in vague terms such as *inflammatory atypia* or *benign atypia*, etc., terms that were used inconsistently and variably by cytologists. With implementation of the Bethesda System, ASCUS became the most common abnormal cervical cytology interpretation; me-

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dian reporting rates for ASCUS hovered in the range of 4% to 5%.[2,3]

ASCUS may have brought some order to the lexicon of laboratory reporting, but the term created a dilemma for clinicians regarding management of women with this cytologic finding. Most women did not have significant high-grade cervical intraepithelial neoplasia (CIN) on workup; however, because of the sheer numbers of women involved, ASCUS was the most frequent cytologic finding preceding histologically confirmed high-grade CIN.[4] Clinicians were divided as to the appropriate management; some favored colposcopy; others, a more conservative approach of follow-up by repeat cytology. As a third option, DNA tests for types of human papillomavirus (HPV) known to cause virtually all cases of cervical cancer offered the possibility of molecular triage.

ASCUS/LSIL TRIAGE STUDY

The ASCUS/LSIL Triage Study (ALTS) was designed and implemented in response to the gynecologic community's request for clinical data to resolve the controversy regarding management of equivocal and low-grade cytologic abnormalities. The multicenter, randomized design compared 3 follow-up strategies for women with ASCUS and LSIL (low-grade squamous intraepithelial lesion): (1) immediate colposcopy regardless of enrollment test results; (2) referral to colposcopy if the enrollment human papillomavirus (HPV) test was positive or missing, or for an HSIL (high-grade squamous intraepithelial lesion) enrollment cytology; and (3) conservative management by repeat cytology at 6-month intervals with referral to colposcopy at the threshold of HSIL.

Between 1996 and 1998, 5060 women with community Papanicolaou test findings of ASCUS (n = 3488) or LSIL (n = 1572) were enrolled and followed for 2 years. At enrollment and at 6-month intervals in follow-up, women underwent pelvic examinations, during which 2 cervical specimens and cervical photographs (Cervigrams, National Testing Laboratories Worldwide, Fenton, MO) were obtained. The first cervical specimen was placed in liquid cytologic medium PreservCyt (Cytyc, Boxborough, MA) and used to prepare a ThinPrep (Cytyc) cytology slide. A 4-mL aliquot of the residual PreservCyt specimen was used for HPV DNA testing (Hybrid Capture 2 [HC2], Digene Corporation, Gaithersburg, MD). The second cervical specimen was collected into specimen transport medium (STM; Digene) for subsequent HPV typing.

Throughout the trial and regardless of study arm, women were referred (or referred again) to colposcopy for a clinical center cytologic interpretation of HSIL and were treated by loop electrosurgical excision procedure (LEEP) for clinical center biopsy histology diagnosis of CIN2 or CIN3. The exit visit at 24 months from the date of enrollment included colposcopy for all women for 2 reasons: to ensure patient safety and to promote complete ascertainment of CIN2, CIN3, or cancer before exiting the study. The main study end point was the diagnosis of histologic CIN3 (n = 535, including 2 cases of adenocarcinoma in situ) or cancer (n = 7) by the Pathology QC group at any time during the 2 years of the trial, termed cumulative CIN3+. Additional details of the trial protocol have been published elsewhere.[5–7]

ASCUS

ALTS found that triage of ASCUS using a single HPV test was at least as sensitive as universal immediate colposcopy in the detection of CIN3+ and spared almost half of women the disadvantages of colposcopy. Immediate colposcopy with biopsy at enrollment detected only about half of cumulatively diagnosed CIN3+. This was a surprising result for the study arm presumed to be the benchmark gold standard for disease detection. It was impossible to accurately separate missed prevalent from newly incident CIN3 for cases diagnosed during follow-up or exit. However, review of complete patient visit histories from the immediate colposcopy arm, and comparisons of cumulative rates between arms, provided strong evidence supporting missed prevalent disease in the majority of cases not found until after the initial colposcopy.

The less-than-perfect sensitivity of colposcopy impacted the efficacy of the other triage strategies that used test results to identify which women should be referred for colposcopy; once referred, the detection of disease was dependent on the colposcopy procedure. Therefore, to evaluate the theoretical optimal test performance—ignoring the imperfect sensitivity of colposcopically directed biopsy—we compared the percent of women with cumulative CIN3+ who were positive by HC2 (at enrollment) with the percent of women who had a repeat cytology at or above the threshold for referral (at enrollment or follow-up).

A single enrollment HPV test was positive in 92% of women with cumulative CIN3+, with the trade-off of referring 53% of women overall. Modeling a conservative management strategy of 2 repeat cytology samples demonstrated very high sensitivity for CIN3+ (95%) if a low threshold of ASCUS+ referred women to colposcopy. However, more women would ultimately be referred (67%) and potentially overtreated. In addition, the multiple follow-up visits required raise concern of loss to follow-up. Repeat cytology with HSIL as the referral threshold had the greatest specificity (only 12% of women were sent to colposcopy), but sensitivity for CIN3+ was only 55% in the trial; assuming perfect colposcopy and biopsy, the sensitivity would still be only 60%.

A cost-effectiveness analysis of the ALTS strategies showed repeat cytology with a threshold of HSIL for referral to colposcopy was the least costly approach per CIN3+

detected but was relatively insensitive (Kulasingam et al, unpublished data). HPV testing was next most cost-effective and was an economically viable strategy, as modeled under a range of scenarios. The cost-effectiveness of HPV testing was even greater (ie, lower cost per CIN3+ detected) among women 30 and above because of the overall lower prevalence of HPV in older women[8] (Kulasingam et al, unpublished data).

The ALTS findings in conjunction with data from other studies[9] provided the basis for the development of the American Society for Colposcopy and Cervical Pathology's Consensus Guidelines for the management of women with abnormal cytology.[10,11] These guidelines provide 3 options for follow-up of women with atypical squamous cells (ASC)-US: colposcopy, HPV testing, or repeat cytology with referral for ≥ASC. However, if a cervical specimen is available (either a residual liquid based or a co-collected specimen) that would obviate the need for another patient visit, then "reflex" HPV testing is the recommended approach.

Natural History of CIN Among Women Referred for ASCUS and LSIL

Cross-sectional studies of ASCUS and LSIL in the literature reported 5% to 20% of women had high-grade CIN at colposcopy. However, such studies underestimate the true risk associated with these cytologic findings because of the limited sensitivity of a single colposcopy to detect disease. The longitudinal 2-year follow-up design of ALTS provided multiple cytologic and visual (Cervigram) screens, as well as an exit colposcopy to ascertain more completely disease associated with ASCUS and LSIL cytology. On the other hand, the relative insensitivity of the CM arm at a threshold of HSIL cytology provided the opportunity for undetected (ie, not biopsied) CIN to regress over 2 years.

Table 1 shows the cumulative diagnoses of CIN 2 and CIN 3+ during the trial for women enrolled with ASCUS. The cumulative rate of detection of CIN3+ was 8% to 9% and did not vary by study arm, even though detection was delayed in the CM arm compared with the other arms. However, detection of CIN2 did vary by study arm; less CIN2 was diagnosed in CM (4.7%) compared with IC (7.9%) and HPV (7.3%) presumably because of regression of some cases of "missed" prevalent CIN2.

Similar trends were found in the population of women with LSIL on study entry (Table 2). The cumulative rate of detection of CIN3+ was 14% to 18% and did not differ significantly by arm. But again, significantly less CIN2 was found in CM (7.6%) compared with the other arms (11%–13%).

The similar cumulative rates for CIN3 across arms, despite delay in detection in CM and therefore the opportunity for regression, support the view of CIN3 as a scientifically rigorous end point for studies of cervical precancer. By contrast, CIN2 is more heterogeneous and includes some morphologically "high-grade-looking" changes that are actually associated with transient infections and are destined to regress.

ASCUS: A Biologic Entity?

Is ASCUS a biologic precursor on the path to true SIL? The cumulative rates of CIN3+ in women referred with ASCUS (8.8%) or LSIL (15.0%) show that the risk associated with ASCUS is in between "negative" and "LSIL" cytology. However, if ASCUS is stratified by the results of the enrollment HPV test, HPV positive ASCUS has a cumulative risk of CIN3+ of 15.2%, comparable to LSIL, while HPV negative ASCUS is associated with a very low risk of CIN3+ (1.4%). Through frequent usage, cytologists and clinicians alike have come to think of ASCUS as a biologic entity. However, as these data show, ASCUS is not one entity but rather represents a mixture of HPV-associated changes biologically similar to LSIL and mimics that are unrelated to HPV.

ASC-Undetermined Significance (US) and ASC-H (Cannot Rule out a High-Grade Squamous Intraepithelial Lesion)

The most recent 2001 Bethesda System realigns the ASCUS category and emphasizes 2 groups of ASC: ASC-US, and ASC-H.[12]

TABLE 1. Cumulative Histologic Diagnosis of CIN2 and CIN3* by Pathology Quality Control Group, Stratified by Study Arm Among Women With ASCUS

	Immediate Colposcopy	HPV Triage	Conservative Management	*P*-Value†	Total
CIN2	92 (7.9%)	85 (7.3%)	55 (4.7%)	0.005	232 (6.7%)
CIN3	97 (8.3%)	101 (8.7%)	108 (9.3%)	0.72	306 (8.8%)
CIN2 or 3	189 (16.2%)	186 (16.0%)	163 (14.0%)	0.26	538 (15.4%)
Total no. women	1163 (100.0%)	1161 (100.0%)	1164 (100.0%)		3488 (100.0%)

*CIN3 includes 2 cases of invasive cancer (one each IC and CM arms) and one case of adenocarcinoma *in situ* (HPV arm).
†*P*-values from χ^2 test for comparison between study arms. Direct comparisons of CIN2 by study arm were statistically significant for Conservative Management versus either Immediate Colposcopy ($P = 0.002$) or HPV Triage ($P = 0.01$).

TABLE 2. Cumulative Histologic Diagnosis of CIN2 and CIN3* by Pathology Quality Control Group, by Study Arm Among Women With LSIL

	Immediate Colposcopy	HPV Triage	Conservative Management	*P*-Value†	Total
CIN2	90 (13.4%)	24 (10.7%)	51 (7.6%)	0.002	165 (10.5%)
CIN3	102 (15.2%)	41 (18.3%)	93 (13.8%)	0.26	236 (15.0%)
CIN2 and 3	192 (28.5%)	65 (29.0%)	144 (21.3%)	0.004	401 (25.5%)
Total no. women	673 (100.0%)	224 (100.0%)	675 (100.0%)		1572 (100.0%)

*CIN3 includes 5 cases of invasive cancer (2 each in IC and CM, and 1 in HPV Triage) and 1 case of AIS in the IC.
†*P*-values from χ^2 test for comparison between study arms. Direct comparison of CIN2 by study arm was statistically significant for Conservative Management versus Immediate Colposocopy ($P < 0.001$).

To evaluate the clinical significance of these diagnostic distinctions, ALTS enrollment liquid-based cytology samples interpreted by the Pathology QC group as ASC-US, ASC-H, and HSIL were compared[13] (Note that because ALTS predated the 2001 Bethesda workshop, slightly different terms of "ASCUS rule out LSIL" and "ASCUS metaplastic" had been used but with criteria largely equivalent to ASC-US and ASC-H, respectively. The 2001 Bethesda terms are used herein.) The relationship between cytologic diagnoses and HC2 positivity showed a striking trend: ASC-US 63%; ASC-H 86%; and HSIL 99% positive for oncogenic HPV. Updating the results of the previous publication to incorporate longitudinal follow-up results, enrollment cytology of ASC-H had a higher positive predictive value (32.4%) for cumulative CIN3+ compared to ASC-US (47.7%). However because ASC-US was 6 times as frequent an interpretation compared with ASC-H, ASC-US preceded the detection of numerically more CIN3 than ASC-H.

As noted above for ASCUS, HPV status stratifies ASC-H risk. HPV positive ASC-H had a 38.3% cumulative risk for CIN3+ compared to 3.4% for HPV negative ASC-H. However, the high frequency of HC2 positive ASC-H limits the clinical utility of HPV triage, and therefore management guidelines recommend colposcopic evaluation for ASC-H cytology.[10,11]

LSIL

Initially in the design of ALTS, the optimistic view held that a significant proportion of cytologic LSIL was either HPV negative (ie, cytologic overcall) or HPV positive but for a low risk HPV type; therefore, testing for only oncogenic HPV types might provide useful triage. However, 84% of women referred with a community diagnosis of LSIL were HPV positive using the HC2 high-risk probe set (probe B), severely limiting triage utility. This finding led to early closure of the LSIL HPV triage arm of the trial.[14] Nor was a strategy of repeat cytology useful for managing LSIL. Strategies that achieved at least 95% sensitivity for CIN3+ resulted in more than 80% of women being referred to colposcopy: specifically, 87% would have been referred based on 2 repeat cytologies using ASCUS as the threshold.[6]

Based on these data, the ASCCP guidelines recommend colposcopic evaluation for women with LSIL. Alternative approaches are outlined for special circumstances in adolescent, pregnant, and postmenopausal women.[10,11]

As noted above, the high HC2 positivity precluded use of HPV testing as a triage tool for LSIL cytology. However, HC2 has been approved by the Food and Drug Administration for use as an adjunct to cytology for primary cervical cancer screening in women 30 and older. Recent screening recommendations include such dual testing as an option,[15] and consequently, the test result combination of HPV-negative LSIL cytology will arise in clinical practice.

A retrospective ALTS analysis of LSIL (Zuna et al, unpublished data) found about 12% associated with nononcogenic HPV types based on type-specific detection using polymerase chain reaction (PCR).[16] Depending on which cytology specimens and which pathologists' interpretation were involved, 5–10% of LSILs were negative for HPV by HC2 and by PCR testing for 27 HPV types, suggesting that these "cases" represented either false-negative HPV test results or false-positive cytologic interpretations of LSIL. Restricting to women aged 30 years and older with HPV-negative LSIL, the cumulative risk of high-grade CIN2 or CIN3+ over 2 years was 0–4% (again depending on the cytology specimen type and the pathologist group): significantly lower than the risk observed for LSIL or HPV positive ASCUS but higher than for HPV-negative ASCUS. Therefore, until additional data are available, such women should be followed according to current guidelines.[10,11]

Diagnostic Reproducibility of Cytology and Histopathology

The Papanicolaou test is considered a "screening" test for cervical abnormalities that are subsequently "diagnosed"

by histologic examination of cervical tissue collected at the time of colposcopy. This reliance on tissue as the diagnostic standard presumes that the combination of colposcopic assessment and histologic evaluation is more reliable than cytology. Apart from issues with colposcopy, does the additional architectural information provided by tissue regarding the involved epithelium translate into more reproducible diagnoses?

In ALTS, cytology and histology specimens were read by experienced pathologists at each clinical center. All slides were then sent to the Pathology QC group, which reviewed specimens masked to the clinical center diagnosis. We were therefore able to compare different pathologists' independently rendered diagnoses of 4948 ThinPreps, 2237 biopsies, and 535 LEEP specimens.[17]

Overall, the interobserver reproducibility was only moderate, regardless of specimen type. Histologic diagnoses, even when based on LEEP specimens, were not significantly more reproducible than cytologic interpretations. Not surprisingly, ASCUS represented the greatest source of disagreement in cytology specimens. Of the 1473 cases interpreted as ASCUS by the clinical center pathologist, 43% were interpreted as ASCUS by QC pathology group. For cases interpreted as LSIL by the clinical center or by the QC pathology group, there was good (68%) concordance of the 2 diagnoses. By contrast, the histologic counterpart of low-grade abnormality, CIN1, was the least reproducible histologic abnormality: only 43% of biopsies read as CIN1 at the clinical centers were considered CIN1 by the QC pathology group, while a similar proportion (41%) were interpreted as negative. While it is likely that the Pathology QC group used more stringent criteria for CIN1 in the context of a research study compared with clinical center pathologists who were responsible for patient care, such stringency does not necessarily translate to accuracy. Notably, most of the clinical center CIN1 cases downgraded to negative by Pathology QC were in fact HPV positive on the correlated PreservCyt sample.[17]

Perhaps not surprisingly, the variability in the interpretation of cytology and pathology specimens is greatest around the positive/negative cut points (ASCUS for cytology, CIN1 for histology), analogous to a low signal-to-noise ratio in quantitative testing. In such situations, re-review of equivocal morphology may not provide increased test accuracy. Rather, a different test modality may provide better clarification. For example, in addition to the obvious clinical utility of HPV testing to stratify risk for women with ASCUS cytology, HPV testing has been proposed as a more objective standard for laboratory quality assurance of cervical morphologic diagnosis.[18]

Postcolposcopy Management

In ALTS, for women referred with LSIL or HPV+ ASCUS, 7% had histologically confirmed CIN3+ at enrollment colposcopy. However, because of imperfect sensitivity of colposcopically directed biopsy, women with <CIN2 at colposcopy (below the threshold for treatment) had approximately 6% risk of subsequently diagnosed CIN3+ within 2 years. Moreover, women with a completely negative initial colposcopy were at approximately the same risk for CIN3+ with 2 years. Moreover, women with a completely negative initial colposcopy were at approximately the same risk for CIN2+ as women with CIN1 at initial colposcopy. Therefore, this distinction was not clinically useful as a basis for decisions regarding subsequent follow-up.

A retrospective analysis of postcolposcopy management strategies (for women with <CIN2 at initial colposcopy) found that HC2 HPV testing at 12 months was 92% sensitive for subsequently detected CIN2+ and would refer 55% of women to colposcopy. Combining cytology and HPV testing did not increase sensitivity but reduced specificity.[19]

HPV DNA TESTING IN ALTS: EVALUATING THE PERFORMANCE OF A MOLECULAR DIAGNOSTIC TEST

The discovery that cervical infections by certain types of HPV (oncogenic HPV) cause virtually all cervical cancer throughout the world[20–22] has led to the development of DNA diagnostics to detect the presence of HPV. As described above, HPV DNA testing using HC2 proved a useful[23] and cost-effective (Kulasingam et al, unpublished data) triage strategy for distinguishing women with ASC cytology and possible underlying precancer from women with ASC at very low risk of disease. In addition, HC2 has been approved by the FDA as an adjunct to cytology screening for women 30 and older. As HPV DNA testing is introduced into clinical practice and with new assays (PCR-based methods, in situ hybridization, and next-generation HC) on the horizon, it is worth summarizing the ALTS experience evaluating the performance of a molecular diagnostic test.

Hybrid Capture 2

HC2 probe set B, the primary HPV DNA assay used in ALTS, is a pooled probe that targets 13 cancer-associated (oncogenic) HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). There is no clinical utility for using probe set A, which targets nononcogenic HPV types 6, 11, 42, 43, and 44, for cervical cancer screening or triage; its use should be discontinued. HC2 (as used in this review) refers to the use of probe set B only.

HC2 can be performed on cervical cell samples collected and stored in either STM or PreservCyt. Use of HC2 in other media has not as yet been FDA approved. Details of HC2 testing are presented elsewhere[24,25] and will only be summarized here. Viral DNA released from cervical cells is hybridized in solution to RNA probes. These hybrids are

captured onto the surface of a well coated with an anti–RNA--DNA hybrid antibody. A second antihybrid antibody conjugated to alkaline phosphatase binds to the immobilizedhybrid, and this "sandwich" is detected by light emission from a chemiluminescent substrate. The relative light units (RLU) are compared with an internal positive control (RLU/PC).

HC2 Test Threshold

In ALTS, 1.0 RLU/PC (the equivalent of ~5000 HPV genomes) was used as the positive/negative cut point based on previous receiver operator characteristic (ROC) analysis using high-grade cervical neoplasia as an end point.[26] Other studies have found that RLU signal strength, a surrogate for viral load,[27,28] was associated with the certainty of abnormal cytology,[26,29,30] suggesting perhaps a higher test threshold could improve specificity without compromising sensitivity.

An evaluation among women with ASCUS in ALTS demonstrated that a higher 10.0 RLU/PC cutpoint would reduce sensitivity for enrollment-detected CIN3+ by 5% and the referral rate by 12%.[8] A second analysis evaluated the performance of HC2 for detection of 2-year cumulative CIN3+ for women with ASCUS cytology.[31] Based on receiver operator curve analysis, the theoretical optimal cut point for test accuracy (giving equal consideration to sensitivity and specificity) for cumulative CIN3+ in women with ASCUS would be 3.76 RLU/PC, which would result in a sensitivity of 89% and specificity of 59%. (By comparison, the sensitivity and specificity of HC2 at the 1.0 RLU/PC threshold were 92% and 51%, respectively.) Given the importance of CIN3+detection in this higher-risk population, the loss in sensitivity, despite the greater overall test accuracy, is unlikely to be clinically acceptable.

While viral load is associated with abnormal pathology, it is not a proxy for severity of the lesion. In fact, CIN1 is associated in some studies with higher viral loads compared with high-grade CIN.[8] An unusually careful, histopathologic analysis of cases of CIN3 in ALTS showed that the size of any surrounding CIN1 was the main determinant of viral load, not the extent of the CIN3.[32] Most CIN3 lesions in ALTS were relatively small.

HC2 Reliability

In addition to test accuracy, diagnostic tests must be reliable (ie, reproducible and adaptable to widespread use in clinical laboratories). In ALTS, the HC2 HPV test results obtained by the clinical center laboratories were compared with results obtained by a reference HPV QC laboratory that retested a random sampling of PreservCyt specimens in an ongoing manner a median of 3 months after collection.[33] The agreement between the 4 clinical laboratories and reference laboratory was excellent, with an overall κ of 0.84 (ranging between 0.78 and 0.89.) The reliability was slightly lower ($\kappa = 0.73$) for specimens in which the cytology was interpreted as negative by the Pathology QC group. By comparison, the reproducibility of cytologic and histologic diagnoses was much less (see above),[17] even when no distinctions of cytologic severity were made and a positive (≥ASCUS) versus negative (<ASCUS) dichotomy was used.

A systematically slightly reduced signal strength and lower percent of positive results was evident for the HPV QC retests compared with each of the 4 clinical centers' results; this may have been the consequence of specimen "aging" after an interval of several months, the nonrandom order of specimen aliquots, or laboratory-specific conditions in testing.

The majority of specimens that first tested positive by the clinical center laboratory but later retested as negative by the HPV QC laboratory had low signal strengths in the first test, in the range of 1 to 3 RLU/PC, which is sometimes referred to as the "gray" zone. The greater number of discordant results in this range is not surprising as all tests become less reliable when as they approach their limits of detection due to a poor signal-to-noise ratio. The unreliability of testing specimens in this gray zone and the uncertain interpretation of discordant test results suggest that it is probably safer to report low positive tests (1–3 RLU/PC) as true positive and manage women accordingly instead of retesting specimens.

Additional Considerations Regarding HC2 and PreservCyt

The introduction of HC2 testing of PreservCyt specimen presents several practical challenges to clinical laboratories. First, HC2 testing of residual PreservCyt specimens is time consuming. Thus, high demand may result in significant backlogs of untested specimens. On the other hand, low demand may result in inefficient small batches and increased testing costs. New strategies to automate testing (Rapid Capture; Digene) and reduced processing time, thereby reducing total testing time, are currently in development and look promising[34] but still require further optimization and validation before coming online. A testing backlog may also result in prolonged storage of residual PreservCyt specimens prior to HC2 testing. FDA currently approves testing out of PreservCyt within 3 months of collection. The impact of PreservCyt storage on HC2 performance needs to be more rigorously evaluated.

Other HPV Assays

DNA amplification assays, such as those based on PCR, are being introduced commercially as alternatives to HC2. PCR testing is also being done as "home brews" by individual laboratories. Establishing clinical performance and test reliability is critical prior to use. It might prove especially difficult for "home brew" to meet the rigorous standards needed to test simultaneously for, and to distinguish between,

the multiple oncogenic HPV types. Rigorous standardization and quality control of specimen preparation, amplification, and amplicon detection are essential for test accuracy and reproducibility. For example, PCR contamination will result in false-positive results and poor specimen processing will result in false-negative results.

Separate cervical specimens were obtained at all patient visits in ALTS for PCR testing for research only (HC2 results were used during the trial for HPV triage management). The HPV QC reference laboratory performed PGMY09/11 L1 consensus primers and reverse line blot hybridization for detection of 38 HPV genotypes.[16] Type specific detection of 13 oncogenic HPV types by PCR showed (1) good agreement with HC2 overall, but less positivity than HC2 and (2) slightly less sensitivity for CIN3+ but higher specificity than HC2 (Castle et al, unpublished data).

A common perception is that PCR-based methods are inherently more sensitive for DNA than non-DNA amplification methods (such as HC2), but this was not borne out in ALTS or in other studies.[35] Cervical specimens are biologically complex and may have inhibitors such that PCR methods may not always achieve the theoretical advantage often demonstrated in model systems.

The somewhat higher clinical sensitivity for HC2 for the detection of CIN3+ compared with PCR assays of the same 13 oncogenic types may also partly be the consequence of the now recognized cross-reactivity of HC2 with nontargeted HPV types[36,37] (Castle et al, unpublished data). The primary "cross-reactive" types are 53, 66, 67, and 71, but the types and the degree of cross-reactivity are more apparent in specimens with abnormal cytology, presumably due to higher viral load of these untargeted (cross-reactive) types. This added pickup of cases comes at the cost of poorer specificity and lower overall accuracy and therefore increased referral of ASCUS women to colposcopy. Two possible explanations as to why cross-reactivity might improve sensitivity include (1) these types, especially HPV66,[21] may occasionally cause CIN3; and/or (2) the detection of infections by more strongly carcinogenic types that were missed by PCR. We observed evidence for both explanations. Therefore, the development of a PCR or any other assay that has equal analytic sensitivity and greater type fidelity than HC2 could potentially result in an equally sensitive, more accurate test that would further reduce the colposcopic referral of women with ASCUS cytology.

HPV Types

HC2 is currently formulated to detect 13 oncogenic HPV types that have repeatedly been found in cervical cancer throughout the world.[20] However, a recent report suggested that additional types such as 26, 53, 66, 73, and 82 should be considered oncogenic.[21] To address the potential clinical utility for detection of CIN3+, an ROC analysis examining stepwise addition of types detected by PCR was performed using ALTS data.[38] The simulated addition of HPV types to a theoretical assay found that the most accurate ASCUS triage test (considering both sensitivity and specificity as equally important) would contain only 8 types (HPV16, 31, 52, 58, 33, 35, 45, and 18); however, such a test would have an unacceptably low sensitivity of 81% for CIN3+, with a specificity of 70%. A 13-type test (mirroring the types targeted by HC2) had a higher sensitivity of 87% but with decreased specificity of 56%. The addition of other candidate oncogenic types only minimally increased sensitivity while significantly lowering specificity. This analysis suggests that it may be ill advised to add additional HPV types to the currently targeted 13 oncogenic types.

Finally, although adding HPV types to current pooled tests has little added clinical utility for triage of women with ASCUS cytology, distinguishing women infected with HPV16 may have clinical implications. Among women with either ASCUS or LSIL cytology, HPV16 DNA–positive women had ~5-fold greater risk of CIN3+ within 2 years, with an absolute risk of 30% to 40%, compared with women positive for other oncogenic HPV types.[39] HPV16 DNA positivity was the single most important predictor of a CIN3+ diagnosis within 2 years; among these women, the further cytologic distinction of ASCUS versus LSIL, or age stratification, were uninformative.

Type-specific testing for HPV16 may be clinically useful, either as a single-type test for women with LSIL cytology or in tandem with a pooled test for the dozen or so other oncogenic HPV types in the context of ASCUS cytology triage.

Clarifying ASCUS Using HPV Typing

To further explore patterns of HPV in women with ASCUS cytology, we used both HC2 and PCR HPV testing results to subclassify women hierarchically according to risk (HPV risk status): HPV16 positive > oncogenic HPV positive, but HPV16 negative > nononcogenic HPV positive, but oncogenic HPV negative > HPV negative. We restricted the analysis to women without CIN2+ at any point (to eliminate any disease related effects).

Overall HPV DNA detection (positive/negative) varied by clinical center (Table 3) and age (Figure 1), highlighting the poorly reproducible nature of ASCUS as a borderline or threshold interpretation.[17] ASCUS was more likely to be HPV DNA negative in older than in younger women, which suggests that there are changes with age that mimic HPV-induced cytologic changes. However, among those women who were HPV positive, the relative fractions of women with

TABLE 3. HPV DNA Testing Results for All Women Referred for an ASCUS Cytology and for Women With ASCUS Cytology and <CIN2 Histology

		All Women		HPV DNA Positive Women Only		
	n	HPV DNA Neg	HPV DNA Pos	HPV16(+)	Oncogenic HPV(+)/HPV16(−)	Non-Oncogenic HPV(+)
ASCUS	3483	1202	2281	501	1386	394
		34.5%	*65.5%*	*22.0%*	*60.8%*	*17.3%*
ASCUS, <CIN2	3038	1182	1856	294	1187	375
		38.9%	*61.1%*	*15.8%*	*64.0%*	*20.2%*
Center 1	982	287	695	114	452	129
		29.2%	*70.8%*	*16.4%*	*65.0%*	*18.6%*
Center 2	403	158	245	42	147	56
		39.2%	*60.8%*	*17.1%*	*60.0%*	*22.9%*
Center 3	645	399	246	38	155	53
		61.9%	*38.1%*	*15.4%*	*63.0%*	*21.5%*
Center 4	1008	338	670	100	433	137
		33.5%	*66.5%*	*14.9%*	*64.6%*	*20.4%*
		$P < 0.00005$			$P = 0.7$	

HPV DNA testing results for women with ASCUS Cytology and <CIN2 histology were also stratified by clinical center.

Row percentages (in italics) are below the numbers; row percentages for "HPV DNA Positive Women only" are calculated using the total number of HPV DNA positive women as the denominator.

Pearson χ^2 tests were used to test for differences between clinical centers in detection of HPV DNA and in HPV status among HPV DNA positives and the results are shown at the bottom of the table.

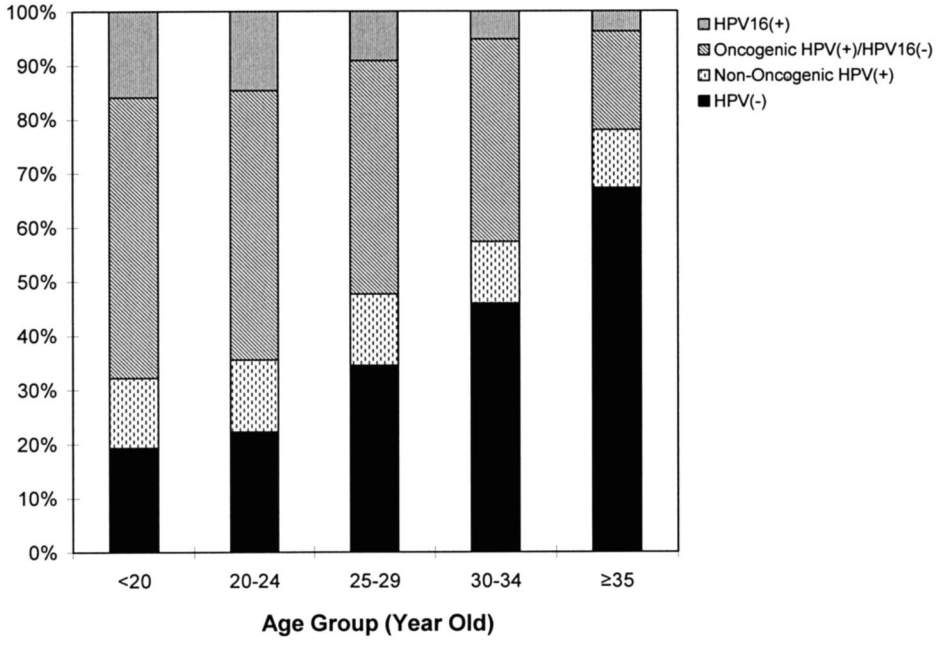

FIGURE 1. HPV DNA status in women with ASCUS cytology and less than histologically confirmed CIN2 by age group are presented. Differences by age were statistically significant ($P_{Trend} < 0.0005$) for all women and for HPV DNA–positive women.

HPV16, other oncogenic types, and nononcogenic types did not vary much by center.

HPV DNA testing of women with ASCUS from lower-risk and/or older populations should result in relatively greater reduction in referral rates than in higher-risk populations.

CONCLUSIONS

The results from ALTS have provided the basis for the development of guidelines for managing women with ASCUS and LSIL cytology. The longitudinal follow-up design of the study provided more complete ascertainment of

disease to better understand the performance characteristics of the various testing modalities. No one test was completely sensitive for detection of cervical precancer and cancer. Even the presumed "gold standard" of colposcopically directed biopsy was associated with a significant proportion of false negatives. And while each test has its own performance characteristics and limitations, cytology, histology, and HPV testing all demonstrate the lowest reproducibility around the test's negative/positive threshold (ASCUS, CIN1, and 1.0 RLU/PC, respectively) where the signal:noise ratio is too low to allow consistent discrimination. In ongoing analyses, we continue to explore the limitations and performance of colposcopic and visual assessment of the cervix.

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